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Advantage of tacrolimus/mycophenolate mofetil regimen for cytotoxic T cell-mediated defence and its inhibition by additive steroid administration in high-risk liver transplant recipients

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Summary

Our previous work revealed that the recipients with the highest pre-existing numbers of $CD8^+$ effector T cells (T_E) [hyperparathyroidism $(HPT)_E$ recipients] occupied approximately 30% of adult transplant recipients performed in our hospital. HPT_E recipients demonstrated very poor clinical outcome compared with the remaining 70% of recipients with the lowest pre-existing T_E (LPT_E recipient). This study aimed to clarify the best combined immunosuppressive regimen related to function of cytotoxic T lymphocytes (CTLs) for HPT_E recipients. Eighty-one HPT_E recipients were classified into three types, according to the immunosuppressive regimens: type 1, tacrolimus (Tac)/glucocorticoid (GC); type 2, Tac/mycophenolate mofetil (MMF)/GC; and type 3, Tac/MMF. Frequencies of severe infection, rejection and hospital death were the highest in types 1 and 2, whereas the lowest occurred in type 3. The survival rate in type 3 was the highest (100%) during follow-up until post-operative day 2000. Regarding the immunological mechanism, in type 1 T_E perforin and interferon (IFN)-γ were generated through the self-renewal of $CD8^+$ central memory T cells (T_{CM}), but decreased in the early post-transplant period due to marked down-regulation of interleukin (IL)-12 receptor beta-1 of T_{CM}. In type 2, the self-renewal T_{CM} did not develop, and the effector function could not be increased. In type 3, in contrast, the effectors and cytotoxicity were correlated inversely with IL-12R$\beta1^+$ T_{CM} levels, and increased at the highest level around the pre-transplant levels of IL-12R$\beta1^+$ T_{CM}. However, the immunological advantage of Tac/MMF therapy was inhibited strongly by additive steroid administration.

Keywords: central memory T cells, highest pre-existing effector T cells, IL-12Rβ1+ cells, immunosuppressive regimen, living donor liver transplantation

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Introduction

Our previous work revealed that recipients with the highest pre-existing numbers of $CD8^+$ effector T cells (T_E) [hyperparathyroidism $(HPT)_E$ recipients] before living donor liver transplantation (LDLT) occupied approximately 30% of adult transplant recipients performed in our hospital. HPT_E recipients demonstrated very poor clinical outcome compared with the remaining 70% recipients with the lowest pre-existing T_E (LPT_E recipients), and were referred to as high-risk recipients [1,2]. Regarding the detrimental mechanism, it has been suggested that the $CD8^+$ T cell functions in HPT_E recipients had already been activated fully in the circulating T_E prior to LDLT [3]. Moreover, the highest activation was inhibited excessively by an inhibition of interleukin (IL)-2 production following tacrolimus (Tac) administration, and the $CD8^+$ T cells lacked most of the effector functions characteristic of activated cytotoxic T lymphocytes (CTLs), similar to activation-induced non-responsiveness (AINR) reported by Mescher *et al.* [4]. In addition, the pre-existing augmented T_E, primed by previous life-long exposure to microbial or viral infection, may potentially cross-react with allogeneic major histocompatibility complex molecules through allogeneic endothelial cells during infiltration in the graft, resulting in allograft destruction (so-called 'heterologous immunity' [5]).

Therefore, we have been continuing to search for immunosuppressive regimens (ISR) capable of improving clinical outcomes for HPT_E recipients.

Immediately after LDLT, the largest numbers of donor-specific alloantigens presented by mature dendritic cells (DCs) [6–8] are released from the allograft, and the $CD8^+$ T cells of the recipient are primed strongly by the alloantigen through the direct pathway via native major histocompatibility complex (MHC) molecules expressed on graft-associated antigen-presenting cells (APC) [9]. In those processes, the most important role of $CD8^+$ T cells is to respond to alloantigens or microbial antigens at an early period. The effective immune responses depend, to a large extent, upon effective T_E (the so-called CTLs) induction, which lyses sensitized or infected cells. At the height of the early immune response, perforin is expressed predominantly by $CD8^+$ CTL [10], indicating a key effector molecule for T cell-mediated cytolysis.

A $CD8^+$ T cell clone is stimulated initially by the first encounter with alloantigen through the T cell receptor (TCR)/CD27 pathway in the secondary lymphoid tissue, leading to the processes of the self-renewing of $CD8^+$ central memory T cells (T_{CM}) [11,12]. Thereafter, signals initiate their differentiation to $CD8^+$ effector cells (T_E) that migrate to the periphery to eliminate alloantigens.

From this perspective, the most important sequence of events after LDLT is how the effector function of CTLs can be generated efficiently at an early period. The accumulated data demonstrated that the development of effector function requires a third signal that can be provided by interleukin (IL)-12 [13–15]. Recently, our work demonstrated that early coupled up-regulation of initial priming (IP) of IL-12Rβ1 of T_{CM} and T_E (IP-12R $T_{CM}T_E$) plays a crucial role in determining a better clinical outcome after LDLT [3]. Consequently, the immune responses after LDLT were markedly different, depending on whether post-transplant levels of IL-12Rβ1^+ T_{CM} were above or below the pre-transplant levels.

Until now, combined therapy of Tac and glucocorticoid (GC) in our hospital has been administered usually as the first choice. Recently, we have been continuing to search how any immunosuppressive regimen can up-regulate IL-12Rβ1^+ T_{CM}, perforin molecules and interferon (IFN)-γ efficiently with respect to CTL function in HPT_E recipients, and to clarify the mechanism to prevent infections and acute cellular rejection (ACR).

Patients and materials

Patients

We performed standard LDLT [16] in 350 adult patients between December 2002 and July 2009 at Kyoto University Hospital. Among these, 134 recipients without immunological study were excluded from this study. Among the remaining 216 adult patients, an immunological study was performed in 81 (37·5%) of the consecutive cohort of HPT_E recipients. To select HPT_E recipients, all the recipients were classified into the following three groups according to hierarchical clustering [17] by preoperative $CD8^+$CD45 isoform profile, as reported previously [1,2]: group I (naive-dominant); group II (effector memory-dominant); and group III (effector-dominant). Group I was referred to as the recipients with the lowest numbers of pre-existing T_E (LPT_E recipients), whereas group III, with the highest numbers of pre-existing T_E, was referred to as HPT_E recipients.

Written informed consent was obtained from the recipients before starting the study, which was approved by the Ethics Committee of Kyoto University Hospital and conducted in accordance with the 1975 Declaration of Helsinki, as revised in 1996.

Immunosuppression

Tac/GC regimens were administered to most recipients until May 2006. Ten years ago, we noted the beneficial effects of Tac/MMF regimen up-regulating IL-12Rβ1 of the CCR7-positive subsets (CPS: T_N, T_{CM} and T_{DP+}), and thereafter, Tac/MMF regimens were employed mainly for HPT_E recipients (non-random selection). The immunological follow-up was the period until their discharge.

Eighty-one HPT_E recipients were classified into three types according to the immunosuppressive maintenance regimens: type 1, Tac/GC; type 2, Tac/MMF/GC; and type 3, Tac/MMF. In the three types, methylprednisolone (10 mg/kg) was administered just before the start of graft reperfusion [18]. In all the types, the initial immunosuppression regimen after LDLT was Tac or cyclosporin A administered from post-operative day (POD) 2. The target whole-blood trough level was adjusted between 10 and 15 ng/ml during the first 3 weeks and approximately 10 ng/ml at the end of the first month; in the out-patient stages, it was maintained between 5 and 10 ng/ml. In types 1 and 2, the initial dose of steroids was reduced rapidly, and they were withdrawn totally by 3–6 months after LDLT. Methylprednisolone (1 mg/kg) was given intravenously for 3 days, starting on POD 1, after which methylprednisolone (0·5 mg/kg) was given for the next 3 days. On POD 7, methylprednisolone was reduced to a daily maintenance level of 0·3 mg/kg, given orally. In types 2 and 3, MMF (0.5–1 g every 12 h) was administered within 24 h after LDLT through the intestinal tube. Thereafter, the recipients were switched to oral MMF (0·5–0·75 g every 12 h). For ABO-incompatible LDLT, the protocol using rituximab prophylaxis and prostaglandin E_1 through the hepatic artery and systemic cyclophosphamide followed by MMF was performed in addition to the standard protocol [19].

Acute graft rejection, infection and tissue typing

The measurements were evaluated employing the methods reported previously [1].

Flow cytometry

We examined peripheral blood mononuclear cells (PBMCs) from each recipient. Sample analyses were performed within 24 h after sampling in all cases. In all recipients, the blood samples were taken and analysed every morning on PODs 0, 5, 8, 12, 20, 28, 32 and 40. Thereafter, the blood samples were taken every 7–10 days until discharge or hospital death. The PBMCs were stained with monoclonal antibodies, as reported previously [1].

Flow cytometric measurement of IFN-γ production was performed after previous stimulation, as described previously [1]. Intracellular perforin in $CD8^+$ cells was measured without previous stimulation, according to the previously reported method [1].

Expression of IL-12 receptors was determined using R-phycoerythrin (PE)-conjugated anti-IL-12Rβ1 and IL-12Rβ2 (BD Biosciences, San Diego, CA, USA). IL-12Rβ1$^+$cells, perforin and IFN-γ were measured after the classification of $CD8^+$ T cells into three subsets, as reported previously [20]. The IFN-γ of $CD8^+$T cells is produced from T cytotoxic-1 (Tc1) cells, similar to a T helper type 1 (Th1)-like cytokine pattern.

Perforin-T_E* and IFN-γ-T_E* were expressed as the absolute proportion of perforin or IFN-γ specific for T_E within $CD8^+$ T cells (T_{CD8}).

Evaluation of post-transplant immune status

As a measure, the proportion of variables immediately before LDLT was subtracted from the proportions at various time-points after LDLT, and expressed as the percentage difference [21].

Statistical analysis

Overall survival was defined as the time from surgery until death from any cause. Survival curves were estimated using the Kaplan–Meier method. Association between the factors and the prognosis were examined with the log-rank test in univariate analyses.

Comparisons of continuous variables between types were performed by applying Student's *t*-test and analysis of variance. Comparisons for proportions between types were undertaken using Fisher's exact test or the χ^2 test. All statistical tests were two-tailed. Significance was defined as $P < 0{\cdot}05$.

Results

Characteristics of the three types classified according to immunosuppressive maintenance regimens

Table 1a shows the clinical analyses of 81 HPT_E recipients classified into the three types based on ISR. Preoperatively, there was no difference in recipient ages, numbers of human leucocyte antigen (HLA) mismatches and risk of cytomegalovirus (CMV) (donor$^+$/recipient$^-$) among the three types. Their clinical status according to the Model for End-Stage Liver Disease (MELD) score [22] was significantly higher in types 2 and 3 than in type 1. The numbers of ABO-incompatible LDLT were highest (53·6%) in type 2, intermediate (21·6%) in type 1 and 0% in type 3. The ratios of the graft weight/body weight were significantly higher in type 1 than in types 2 and 3. Among primary diseases, the total numbers of hepatitis C virus (HCV)-related cirrhosis comprised 44·4%, hepatitis B virus (HBV)-related cirrhosis comprised 18·5% and primary biliary cirrhosis comprised 13·6%.

Table 1b shows the frequencies of post-transplant episodes until their discharge in the three types.

In type 1 recipients, the infection sites were: blood in 40% (such as *Pseudomonas aeruginosa*, methicillin-resistant coagulase-negative staphylococci (MRCNS) and methicillin-resistant *Staphylococcus aureus* (MRSA), urinary tract in 20%, infection associated with the central venous line, other catheter and drain in 90%, bile in 10% and respiratory tract in 10%. Before LDLT, two recipients showed spontaneous bacterial peritonitis (SBP) and three other recipients had candida infection and pneumonia.

In type 2 recipients, the infection sites were: blood in 66·7% (*S. epidermides* and *Enterococcus faccium*), ascites in 33% (*E. cloacae*), central venous catheter in 33% (MRCNS) and trachea in 33% (*Candida albicans*). These infections were not detected before LDLT.

The lowest infectious complications were in type 3 recipients.

Severe sepsis (SS) and SS/multiple organ dysfunction syndrome (MODS). The incidence of SS/MODS was highest in type 1 and lowest in types 2 and 3.

ACR. The incidence of biopsy-proven acute cellular rejection was 16·2% in type 1, 27·6% in type 2 but 13·3% in type 3.

Hospital deaths. The main causes of hospital death were life-threatening infectious complications, followed by surgical complications, acute rejections and other factors.

In type 1 recipients, there were 10 hospital deaths (27·0%) among 37 recipients. Those recipients were complicated seriously by cerebral bleeding in one, septic shock in two, SS/MODS in two, biliary duct stricture or leakage in two, hepatic artery thrombosis in one and abdominal bleeding associated with an emergency operation in two recipients.

In type 2 recipients, there were three hospital deaths (10·3%) among 29 recipients. Those recipients were complicated with serious SS/MODS in two and hepatic artery thrombosis in one recipient. In type 3, there was no hospital death.

Frequencies of infection and rejection in ABO-incompatible and ABO-compatible recipients in types 1 and 2

In type 1, frequencies of infection in ABO-incompatible LDLT were 100% for bacteria, 75·0% for viruses and 37·5%

Table 1. Clinical analysis of effector T cell (T_E) [hyperparathyroidism $(HPT)_E$] recipients classified into the three types based on immunosuppressive regimens

(a) Clinical analysis

Regimens	Type 1	Type 2	Type 3	Total	*P*-value
Number of recipients	37	29	15	81	
Age (male/female)	51 ± 13	56 ± 7	55 ± 10	54 ± 11	0·0939*
Male/female	15/22	11/18	8/7	34/47	0·6003†
MELD score	16 ± 8	21 ± 10	21 ± 9		0·0509*
HLA-mismatch numbers (≥ 3)	9	14	5	28	0·1264†
ABO-incompatible LDLT	8	15	0	23	0·0007†
Risk of CMV	2	2	0	4	0·6058†
Donor (+)/recipient (−)					
GW/BW ratio	1·146	0·990	0·994		0·0155†
Primary disease					0·2392†
Viral hepatitis C	19	12	5	36	
Viral hepatitis B	5	8	2	15	
Primary biliary cirrhosis	2	6	3	11	
Primary sclerosing cholangitis	1	0	1	2	
Autoimmune hepatitis	1	2	0	3	
Fulminant hepatic failure	1	0	0	1	
Biliary atresia	2	0	0	2	
Other	6	1	4	11	

(b) Post-operative complications with different immunosuppressive regimens

Regimens	Type 1	Type 2	Type 3	Total	*P*-value
Infection	*n* (%)				
Bacteria	31 (83·8)	22 (75·9)	3 (20·0)	56 (69·1)	< 0·0001†
CMV	23 (62·2)	18 (62·1)	1 (6·7)	42 (51·9)	0·0005†
Fungus	10 (27·0)	4 (13·8)	0 (0)	14 (17·3)	0·0540†
SS/MODS	13 (35·1)	3 (10·3)	0 (0)	16 (19·8)	0·0044†
Rejection	10 (27·0)	9 (31·0)	2 (13·3)	21 (25·9)	0·4369†
ACR	6 (16·2)	8 (27·6)	2 (13·3)	16 (19·8)	0·4056†
Chronic	2 (5·4)	1 (3·4)	0 (0)	3 (3·7)	0·6432†
Humoral	2 (5·4)	0 (0)	0 (0)	2 (2·5)	0·2954†
Hospital death	10 (27·0)	3 (10·3)	0 (0)	13 (16·0)	0·0321†

**P*-values based on analysis of variance; †*P*-values based on the χ^2 test. Values are expressed as the mean ± standard deviation. GW/BW ratio = graft weight/body weight ratio; ACR = acute cellular rejection; CMV = cytomegalovirus; HLA = human leucocyte antigen; LDLT = living donor liver transplantation; MELD = Model for End-Stage Liver Disease; SS/MODS = severe sepsis leading to multiple organ dysfunction syndrome.

for fungi compared to 75·9, 55·2 and 24·1% in ABO-compatible LDLT, respectively. Furthermore, the rejection rates were 37·5% in ABO-incompatible LDLT compared with 24·1% in ABO-compatible LDLT.

In type 2, frequencies of infection were 73·3% for bacteria, 66·6 for viruses and 20·0% for fungi in ABO-incompatible LDLT, compared to 78·6, 55·0 and 7·1% in ABO-compatible LDLT, respectively. Furthermore, the rejection rates were 20·0% in ABO-incompatible compared with 42·9% in ABO-compatible LDLT.

Consequently, it seems likely that the inclusion of ABO-incompatible LDLT did not affect the results in this study strongly, except for the Kaplan–Meier survival estimates.

In type 3, there was no ABO-incompatible LDLT.

Incidence of surgical complications

The incidence of surgical complications are as follows:

- Type 1: hepatic artery thrombosis, two; hepatic artery bleeding, four; portal vein thrombosis, one; hepatic vein stenosis, one; biliary duct stricture, three; biliary leakage, four; intestine perforation, one; and cerebral bleeding, one.
- Type 2: hepatic artery thrombosis, one; hepatic artery bleeding, one; biliary duct leakage, two.
- Type 3: no complication.

The frequencies of surgical complications were highest among type 1 recipients.

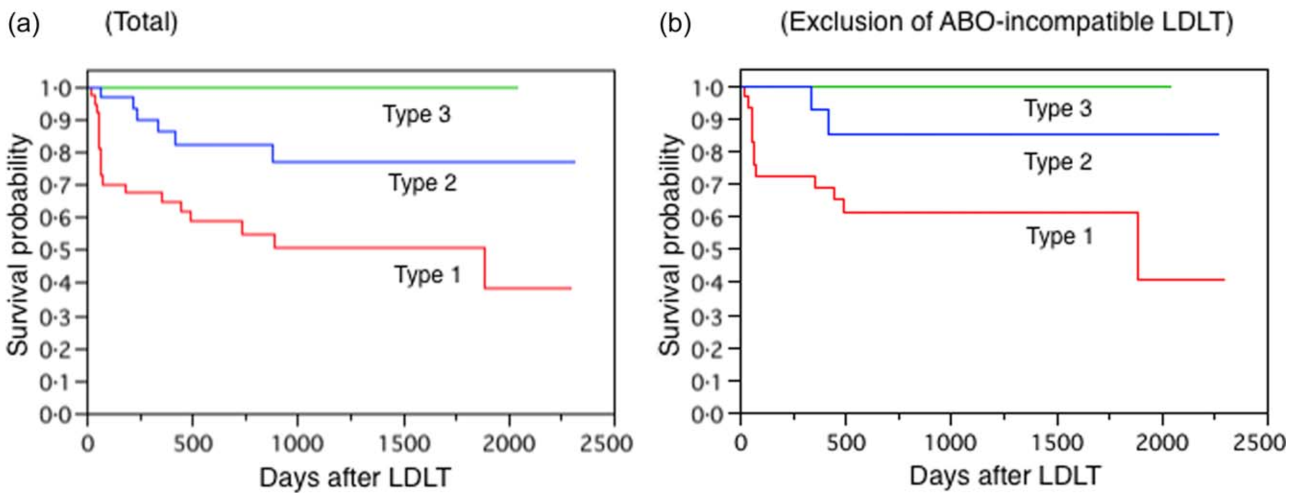

Fig. 1. Kaplan–Meier estimates in each effector T cell (T_E) [hyperparathyroidism $(HPT)_E$] recipient including (a) and excluding (b) ABO-incompatible living donor liver transplantation (LDLT).

Kaplan–Meier estimates in the recipients immunosuppressed by three regimens

These estimates have potential limitations, because of retrospective review survival.

Figure 1 (left) shows the Kaplan–Meier survival curve in all HPT_E recipients immunosuppressed according to the three different regimens. The survival rate in type 3 was highest (100%) during follow-up until POD 2000. In type 2, the survival rate decreased to approximately 80% on POD 750 and then remained at that level beyond POD 2000. In type 1, in contrast, the survival rate decreased below 70% on POD 500 and then to approximately 40% after POD 2000. When the entire follow-up period was compared by a Kaplan–Meier analysis and log-rank test, survival was significantly ($P = 0{\cdot}0021$) lower in type 1 than in types 2 and 3. There was no significant ($P = 0{\cdot}1373$) difference between types 2 and 3. Figure 1 (right) shows the Kaplan–Meier survival curve of recipients excluding ABO-incompatible LDLT (right) receiving the three different regimens. The survival curves in types 1 and 2 were increased slightly by approximately 10%, but the patterns in the three types were generally similar to those including ABO-incompatible recipients. The survival estimates were significantly ($P = 0{\cdot}0090$) lower in type 1 than in types 2 and 3. There was no significant ($P = 0{\cdot}2153$) difference between types 2 and 3.

Immunological characteristics after LDLT in the three types

Phenotypical and functional changes of $CD8^+$T cells in a typical type 3 recipient

Figure 2 shows changes on flow cytometry in IL-12Rβ1^+cells of the CPS and CCR7-negative subsets (CNS: T_E, T_{EM} and T_{DP-}) as well as T_E, perforin-T_E* and IFN-γ-T_E* after LDLT in a typical type 3 recipient (a 54-year-old female) undergoing LDLT under primary biliary cirrhosis. Severe sepsis occurred during PODs 5–12. Decreased hepatic blood flow was confirmed for 1 week from POD 15 by Doppler evaluation. Biopsy-proven acute cellular rejection was detected on POD 39. She was discharged on POD 52. Figure 2a shows changes in the percentage difference of IL-12Rβ1^+cells of the CPS and CNS after LDLT. IL-12Rβ1^+ T_{CM} were decreased slightly to sub-pretransplant levels during PODs 5–12, due possibly to severe infection, and then increased above the baseline after POD 18. IL-12Rβ1^+ T_{DP+} were increased markedly to higher levels than IL-12Rβ1^+ T_{CM}. Importantly, IL-12Rβ1^+ T_N were increased above the pretransplant levels after LDLT. IL-12Rβ1^+ cells of the CNS remained at a slightly higher level than the baseline after LDLT.

Figure 2b shows changes in the percentage difference of T_E, perforin-T_E* and IFN-γ-T_E* after LDLT. T_E and perforin-TE* increased to approximately 5 percentage points on POD 5, increased to 15 percentage points on POD 12 and then returned to just above baseline. In contrast, IFN-γ-T_E* remained at pretransplant levels after LDLT. T_E (r = –0·681), perforin-T_E* (r = –0·421) and IFN-γ-T_E* (r = –0·806) were generated in inverse correlation with IL-12Rβ1^+ T_{CM}.

Figure 3a,b shows changes in IL-12Rβ1^+ cells of the CPS and CNS after LDLT in three types. Figure [3]c shows changes in T_E, perforin-T_E* and IFN-γ-T_E* after LDLT in three types.

In type 1, IL-12Rβ1^+ cells of T_{CM} and T_{DP+} were decreased markedly to below pretransplant levels during PODs 5–12, and then increased slightly after POD 20 (Fig. 3a, left). IL-12Rβ1^+ T_N was decreased slightly on POD 5, and then increased to near baseline, but increased to 10% on POD 25. IL-12Rβ1^+ T_{CM} was correlated significantly positively with that of T_N (r = 0·438) and T_{DP+}

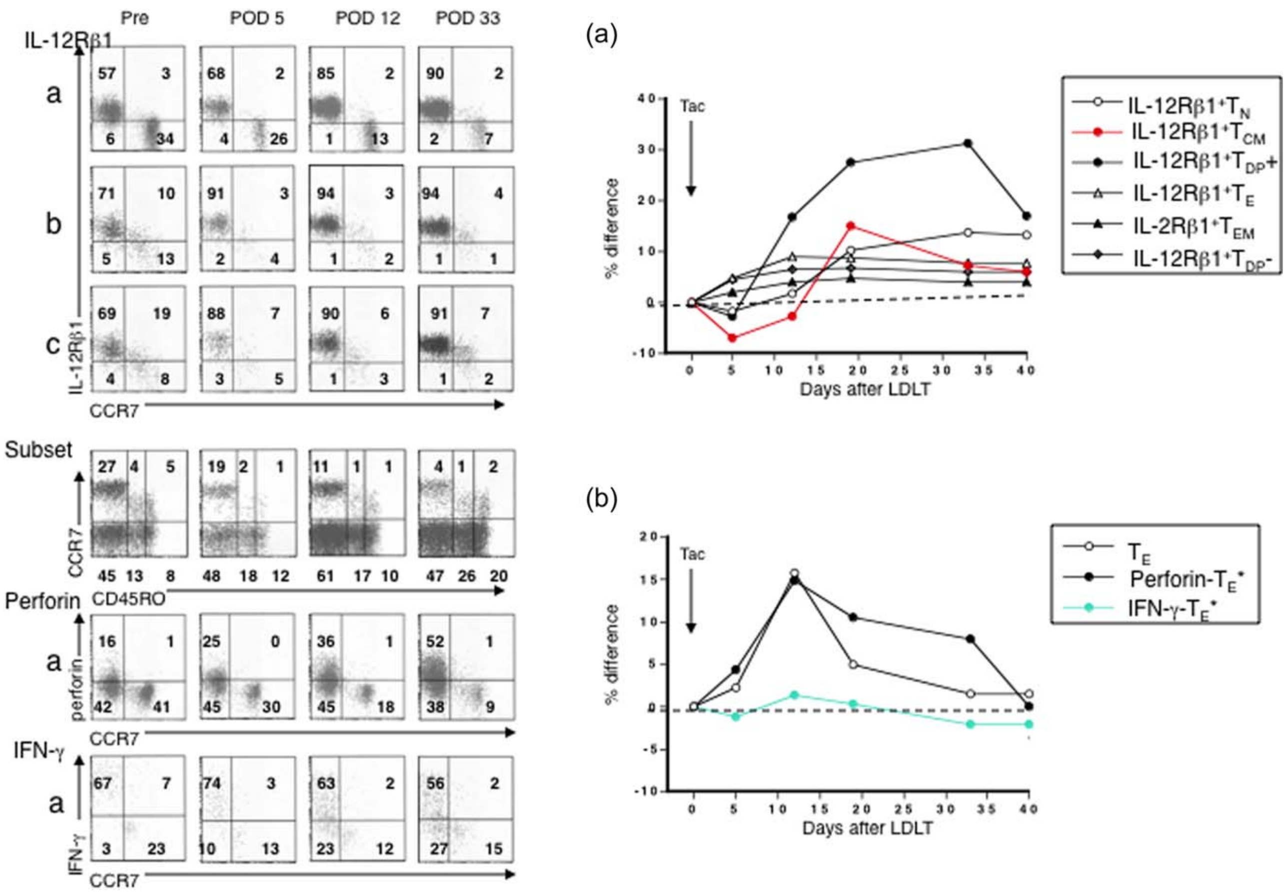

Fig. 2. Flow cytometric assay of the changes in interleukin (IL)-12Rβ1$^+$cells, effector T cells (T_E), perforin-T_E* and interleukin (IFN)-γ-T_E* after living donor liver transplantation (LDLT) in a typical type 3 recipient. IL-12Rβ1-, perforin- and IFN-γ-expressing cells superimposed on double-staining of each of the variables and CCR7 in gated CD8$^+$CD45RO$^-$ cells (a), gated CD8$^+$CD45RO$^+$cells (b) and CD8$^+$CD45RO^{++}cells (c) [20]. IL-12Rβ1$^+$ central memory T cells (T_{CM}) was measured on the gated CD8$^+$CD45RO^{++} cells and perforin and IFN-γ were measured on the gated CD8$^+$CD45RO$^-$ cells. For CD8$^+$ T cell subsets, the lymphocytes were stained using peripheral blood nuclear cell monoclonal antibodies to CD45RO and CCR7. Double-staining for CD8$^+$CCR7/CD45RO on gated lymphocytes identified six subsets of CD8$^+$ T cells: naive (T_N) (CD45RO$^-$CCR7$^+$), central memory (T_{CM}) (CD45RO$^+$CCR7$^+$), effector memory (T_{EM}) (CD45RO$^+$CCR7$^-$) and T_E (CD45RO$^-$CCR7$^-$). Double-positive (DP$^+$)(T^+_{DP}) (CD45RORACCR7$^+$) and double-negative (DP$^-$) (T^-_{DP}) (CD45RORA-CCR7$^-$), as reported previously [20]. Cells in six segments are presented as the ratio (%).

(r = 0·694). IL-12Rβ1$^+$ CNS was decreased markedly on POD 5, and then increased to baseline, followed by increases to 10% above the baseline after POD 33 (Fig. 3b, left). IL-12Rβ1$^+$ T_{CM} was correlated significantly positively with that of T_E (r = 0·608), T_{EM} (r = 0·695) and T_{DP-} (r = 0·709). Conversely, T_E, perforin-T_E* and IFN-γ-T_E* were decreased markedly below pretransplant levels until POD 12 and then remained at sub-pretransplant levels (Fig. 3c, left).

In Type 2 (Fig. 3a,b, middle), the changes in IL-12Rβ1$^+$cells of the CPS and CNS showed a moderate decrease, with patterns similar to those in type 1. IL-12Rβ1$^+$ T_{CM} was correlated significantly positively with that of T_N (r = 0·717) and T_{DP+} (r = 0·638), T_E (r = 0·536), T_{EM} (r = 0·454) and T_{DP-} (r = 0·423). Conversely, $T_{E,}$ perforin-T_E* and IFN-γ-T_E* were increased slightly above the pretransplant levels until POD 12, and then decreased below the baseline on POD 26, although IFN-γ-T_E* was decreased markedly during PODs 18–26 (Fig. 3c, middle).

In type 3 (Fig. 3a, right), IL-12Rβ1$^+$cells of T_{CM} and T_{DP+} were decreased slightly below pretransplant levels on POD 5 and then increased to above baseline during the post-transplant period, especially IL-12Rβ1$^+$ T_{DP+} (Fig. 3a, right). IL-12Rβ1$^+$ T_N remained at pretransplant levels until POD 5 and then remained at a steady state of approximately 10% above pretransplant levels during the post-transplant period. IL-12Rβ1$^+$ T_{CM} was correlated significantly positively with that of T_{DP+} (r = 0·814) and T_N (r = 0·688). Conversely, T_E and perforin-T_E* were decreased below sub-pretransplant levels until POD 12, and then increased above baseline (Fig. 3c, right).

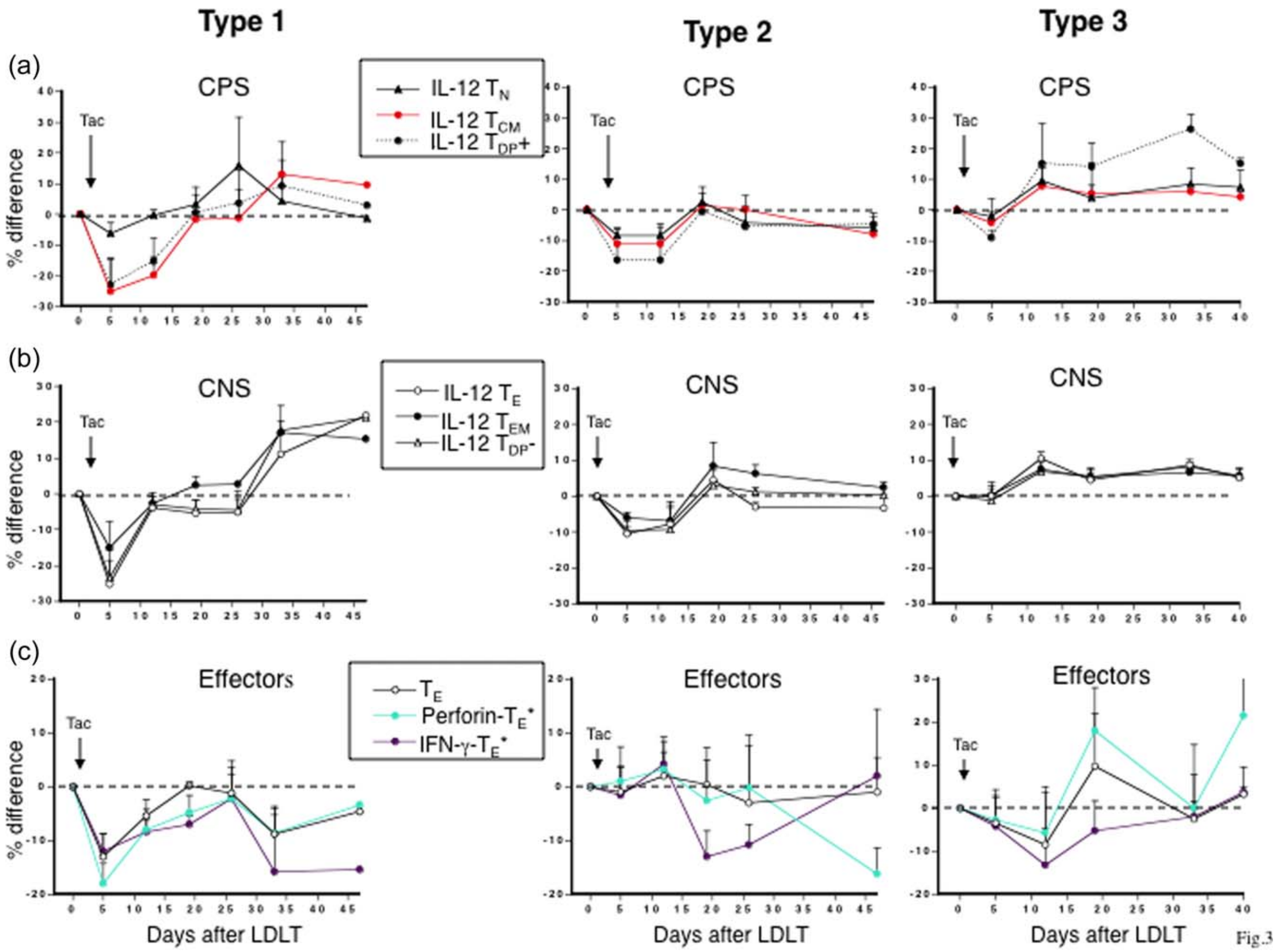

Fig. 3. Changes in the % difference of interleukin (IL)-12Rβ1 in the CCR7-positive subsets (CPS) and central nervous system (CNS) as well as effector T cells (T_E), perforin-T_E* and interferon (IFN)-γ-T_E* after living donor liver transplantation (LDLT) in three types. The IL-12Rβ1$^+$cells, T_E, perforin-T_E* and IFN-γ-T_E* were measured according to the method in Fig. 2.

Changes in the effector function related to the self-renewing T_{CM} in a typical group III recipient receiving type 1 regimen.

Figure 4a shows the flow cytometry (IL-12Rβ1$^+$ T_{CM}, perforin-T_E*, CD8$^+$ T cell subset and IFN-γ-T_E*) of a recipient (a 41-year-old man) undergoing LDLT for HCV-related liver cirrhosis. He showed an uneventful course after LDLT and was discharged on POD 18. The right panel (Fig. 4b) shows changes in the proportion (upper) and percentage difference (low) of IL-12Rβ1$^+$ T_{CM}, perforin-T_E*, T_E and IFN-γ-T_E* after LDLT. The four variables were decreased markedly in parallel below pretransplant levels on POD 5 and then returned to baseline on POD 20. IL-12Rβ1$^+$ T_{CM} was correlated significantly highly with T_E (r = 0·982, P = 0·003), perforin-T_E* (r = 0·987, P = 0·002) and IFN-γ-T_E* (r = 0·871, P = 0·005).

These results suggest that the effectors and cytotoxicity produced by CTLs through the self-renewal T_{CM} were regulated strongly by the IL-12Rβ1$^+$ T_{CM}. However, the extent of effector function was considerably lower in an early period under down-regulation of IL-12Rβ1$^+$ T_{CM} than that under late up-regulation above baseline. It seems likely that the marked down-regulation in the initial priming of IL-12Rβ1$^+$ T_{CM} plays a crucial deleterious role for immune responses and clinical outcomes.

Figure 5 shows changes in IL-12Rβ1$^+$ T_{CM} related to the generation of T_E and perforin-T_E* after LDLT.

In type 1, IL-12Rβ1$^+$ T_{CM} decreased markedly below pretransplant levels during PODs 5–12, and then returned after POD 20 (Fig. 5a, top). T_E and perforin-T_E* decreased after POD 5 and then returned to near the sub-pretransplant levels after POD 20. There was a highly significant positive correlation between IL-12Rβ1$^+$ T_{CM} and T_E or perforin-T_E* (Fig. 5b, top).

In Type 2, IL-12Rβ1$^+$ T_{CM} decreased moderately during PODs 5–12 and then returned to approximately pretransplant levels (Fig. 5a, middle). T_E and perforin-T_E* remained around baseline, followed by decreases in perforin-T_E* on POD 47. There was no significant correlation between IL-12Rβ1$^+$ T_{CM} and T_E or perforin-T_E* (Fig. 5b, middle).

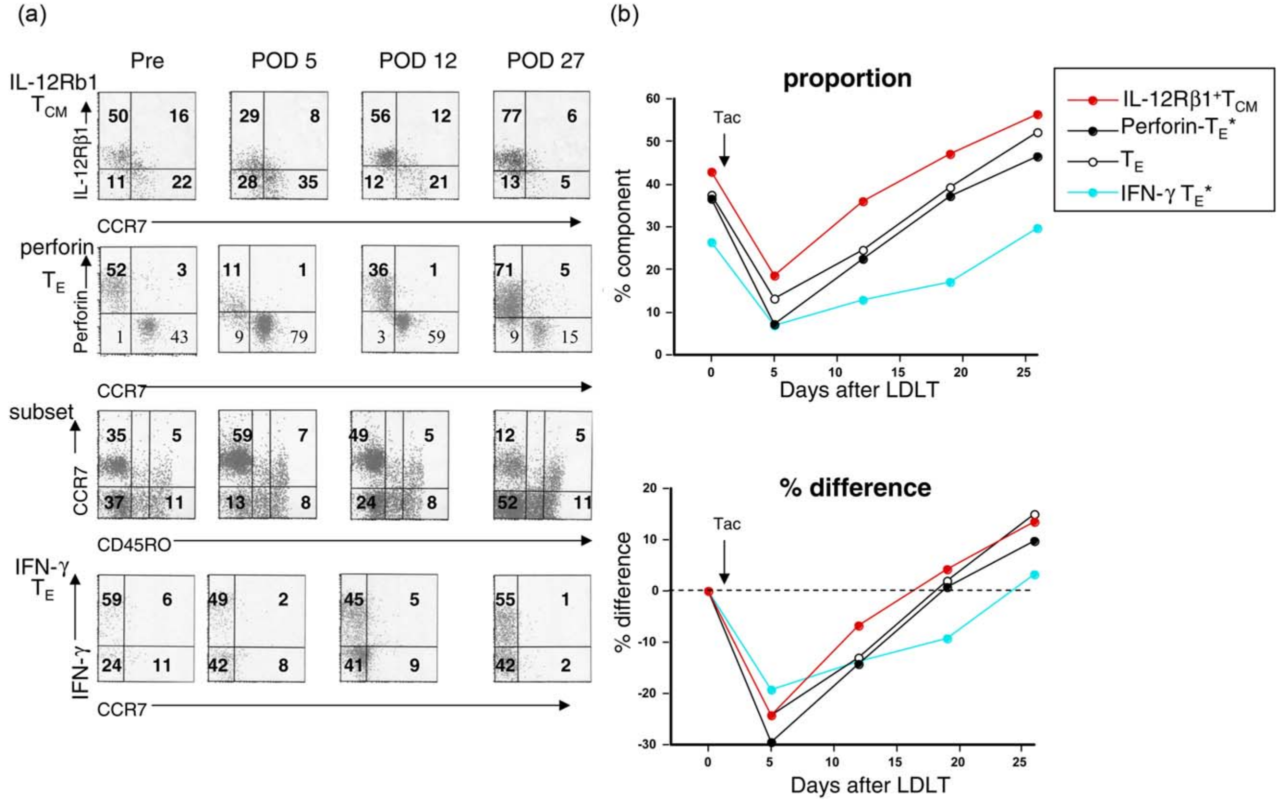

Fig. 4. Changes on flow cytometry in the proportion of interleukin (IL)-12Rβ1$^+$ central memory (T_{CM}), perforin-T_E^*, effector T cells (T_E) and interferon (IFN)-γ-T_E* after living donor liver transplantation (LDLT) in a typical group III recipient (a). Right panel (b): time–course of four variables after LDLT and the correlation among variables. The IL-12Rβ1$^+$ cells, T_E, perforin-T_E* and IFN-γ-T_E* were measured according to the method in Fig. 2. Tac = tacrolimus.

In contrast, in type 3, the IL-12Rβ1$^+$ T_{CM} increased slightly to over baseline during the post-transplant period (Fig. 5a, bottom). T_E and perforin-T_E* remained at sub-pretransplant levels until POD 12, and then increased above baseline. An increase in IL-12Rβ1$^+$ T_{CM} corresponded inversely with decreases in T_E and perforin-T_E*. Importantly, there was a strong significant inverse correlation between IL-12Rβ1$^+$ T_{CM} and T_E or perforin-T_E* (Fig. 5b, bottom). These inverse correlations between effector variables and IL-12Rβ1$^+$ T_{CM} contrasted with the positive correlation in type 1 and no correlation in type 2.

Consequently, in type 1, these results suggest that the effectors and cytotoxicity produced by CTLs through the self-renewal of T_{CM} were regulated strongly by the IL-12Rβ1$^+$ T_{CM}. However, the extent of the effector function was considerably lower at an early period under the down-regulation of IL-12Rβ1$^+$ T_{CM} than that under late up-regulation above the baseline. It seems likely that the marked down-regulation in initial priming of IL-12Rβ1$^+$ T_{CM} plays a crucial deleterious role for immune responses and clinical outcomes. In type 2, there was no significant correlation between generation of T_E and perforin-T_E* and IL-12Rβ1$^+$ T_{CM}, indicating no development of the self-renewal of T_{CM}. In contrast, in type 3, the levels of IL-12Rβ1$^+$ T_{CM} at all time-points were limited within the narrowest range slightly above pretransplant levels. T_E and perforin were generated markedly, along with the approach of IL-12Rβ1$^+$ T_{CM} to pretransplant levels, although MMF inhibits the proliferation of T lymphocytes through the inhibition of inosine monophosphate dehydrogenase [24,25].

Discussion

Immunological characteristics in an early period after LDLT

Early alloimmunity. This sequence of events is strongly dependent upon the following two factors. (1) The largest numbers of donor-specific alloantigens are released from the allograft immediately after LDLT and the CD8$^+$ T cells of the recipient are primed by the alloantigen, and encounter allogeneic endothelial cells during infiltration in the graft. (2) A pre-existing augmented T_E pool cross-reacts strongly with allogeneic major histocompatibility complex (MHC) molecules through allogeneic endothelial cells during infiltration into the graft, resulting in allograft destruction (so-called 'heterologous immunity' [5]). As a result, a

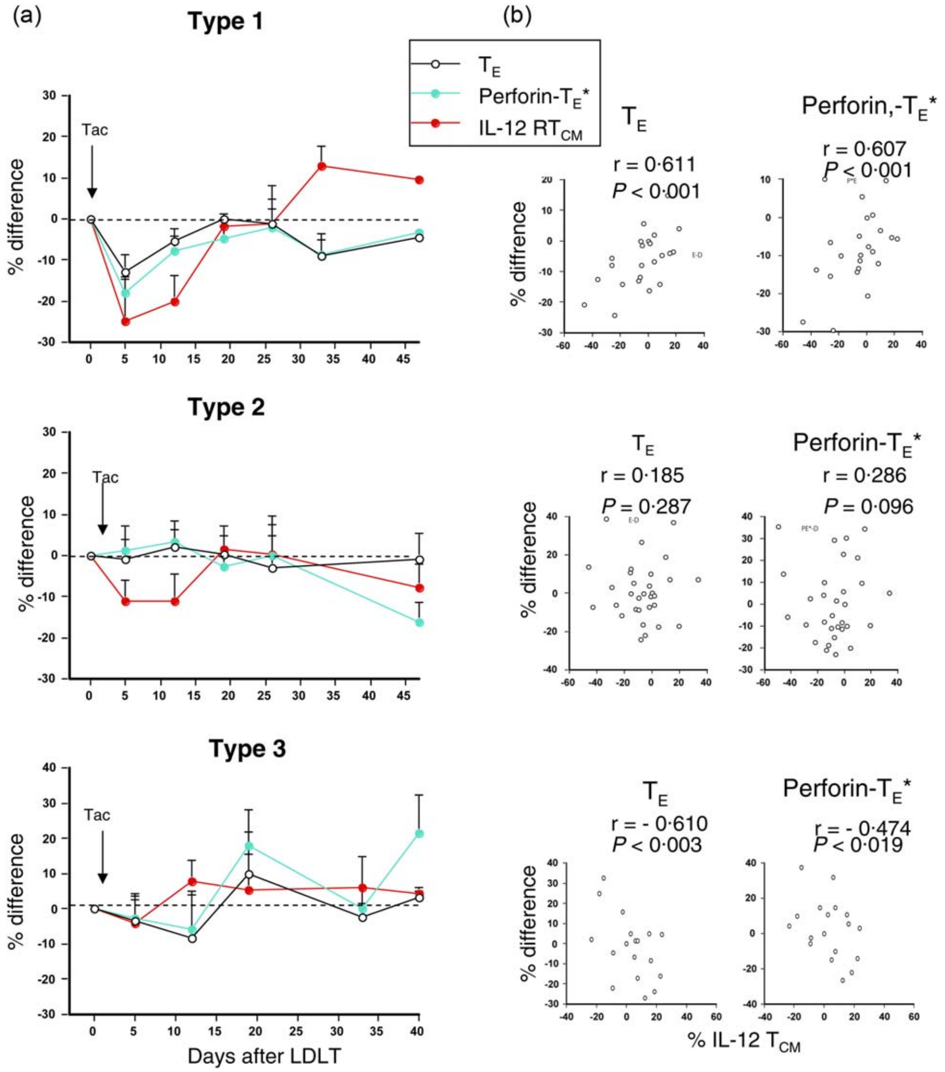

Fig. 5. Changes in the generation of effector T cells (T_E) and perforin-T_E* related to interleukin (IL)-12Rb1$^+$ T_{CM} after living donor liver transplantation (LDLT). The recipients analysed included 13 follow-up cases with type 1, eight cases with type 2 and seven cases with type 3. The IL-12Rb1$^+$ cells, T_E, perforin-T_E*, and interferon (IFN)-γ-T_E* were measured according to the method in Fig. 2. Tac = tacrolimus.

marked and prolonged down-regulation of T_E and perforin-T_E* developed, along with decreases in IP-12R $T_{CM}T_E$.

Infectious immunity. In addition to early alloimmunity, the capacity of effectors and cytotoxicity would be decreased more markedly, leading to the highest frequencies of post-transplant infection, as shown previously [23]. During those processes, sustained T cell stimulation was critically dependent upon a continuous supply of DCs after the development of infection. Many DC precursors would be recruited continuously into inflamed tissues including transplanted allograft and, from there, migrate continuously to the draining lymph nodes, where they arrive endowed with T cell-stimulatory and -polarizing capacities.

Two phases after LDLT

The duration (so-called lag time) required for early coupled up-regulation of IL-12Rβ1 of T_{CM} and T_E over pretransplant levels play a crucial role for better immune responses and clinical outcome after LDLT [3]. Consequently, we classified their post-transplant courses into two phases: first before the lag time, and secondly after the lag time. In the first phase, the marked down-regulation of IP-12Rβ1 $T_{CM}T_E$ before the lag time induced severe suppression of effectors and cytotoxicity of CTLs, and induced marked accumulation of alloantigen-primed antigen within the secondary lymph node tissues. During this time, serious life-threatening complications developed more frequently. In contrast, in the second phase CD8$^+$ effectors and cytotoxicity were up-regulated together with an increase in T_E and perforin-T_E* along with an increase in IL-12Rβ1 of T_{CM} and T_E over pretransplant levels. In these processes, IL-12Rβ1$^+$ T_{CM} played a crucial role in up-regulating IL-12Rβ1$^+$ T_E of the CNS because of their coupling with IL-12Rβ1$^+$ T_{CM} [20]. The lag time was 35 days in type 1, 20 days in type 2 and 0 days in type 3. Hospital deaths more occurred frequently within the first phase in types 1 and 2.

Modulation of effector function of CTLs

Two general proposals have been advanced to account for the development of antigen-stimulated $CD8^+$ T cells [24]. First, clonal expansion mediated by IL-2 is considered to be responsible for generation of the T_{EM} and T_E subsets of antigen-experienced $CD8^+$T cells. Secondly, a stem cell-like capacity for self-renewal could be the basis for the continual generation of effector lymphocytes from the memory pool.

In this study, IL-2 production was inhibited by Tac administration in all recipients, Consequently, a stem cell-like capacity for self-renewal T_{CM} could be the basis for the continual generation of effector lymphocytes from the memory pool.

As shown in Fig. 5, the self-renewal T_{CM} developed in type 1, but T_E, perforin-T_E* and IFN-γ-T_E* were decreased below pretransplant levels in a positive correlation with decreases in IL-12Rβ1^+ cells of the CPS and CNS. In type 2, the self-renewal T_{CM} did not develop during the post-transplant periods, and the effectors and cytotoxicity could not enhance irrespective of the presence of antigens. IL-12Rβ1^+ T_N as an initial IL-12 assist was decreased markedly, resulting in down-regulation of not only an interaction of T_N with mature DCs, but also a cross-reaction of the highest pre-existing numbers of T_E with allogeneic molecules.

In type 3, in contrast, the expression levels of IL-12Rβ1^+ T_{CM} at all time-points were limited within the narrowest range slightly above the pretransplant levels, because MMF inhibits the proliferation of T lymphocytes through the inhibition of inosine monophosphate dehydrogenase [25,26]. T_E and perforin were generated markedly, along with the approach of IL-12Rβ1^+ T_{CM} to pretransplant levels, but the self-renewal T_{CM} could not occur. These results suggest that the restriction of IL-12Rβ1^+ T_{CM} to near pretransplant levels provides a latent multi-potential capability similar to pretransplant immune status, yielding a variety of functional outputs to ensure immunological functioning efficiently. During those processes, the steady-state expression of IL-12Rβ1 of CPS remained above pretransplant levels. IL-12Rβ1^+ T_N was maintained above pretransplant levels during the post-transplant period. This indicates that, in addition to TCR and co-stimulatory molecules, the signal strength of T_N was assisted initially with the up-regulation of IL-12Rβ1^+ T_N. The fully activated signal strength of T_N resulted in an efficient cross-reaction of T_N with allogeneic cells and mature DCs, and promoted the clearance of alloantigens and infectious antigens. In particular, IL-12Rβ1^+ cells of the CNS were increased above pretransplant levels by coupling with IL-12Rβ1^+ T_{CM} during the post-transplant period [20]. In addition, IFN-γ expression was increased to above pretransplant levels, suggesting a deviation towards the stable production of $CD8^+$ Tc1 cytokines, thereby conferring immunity against pathogens.

Conversely, in our hospital, the viral infection rate was 57% (HCV, 38%; HBV, 19%) of total recipients. Compared with HBV and non-viral-infected recipients, HCV recurs in virtually all transplant recipients following otherwise technically successful liver transplantation. Liver transplantation for HCV-related liver failure is followed invariably by acute infection of the allograft. HCV was related closely to moderation of $CD8^+$ effector memory T cells (T_{EM}) after LDLT. We have already demonstrated that plasma HCV-RNA increased rapidly and then peaked, as an initial burst, around POD 25 in group I, at POD 40 in group II and at POD 55 in group III. The initial burst of viraemia was preceded by an increase in the $CD8^+$ T_{EM} pool in 90% of recipients. Those events were suppressed with high expression of $CD8^+CD28^-CD27^-$ subsets [2]. $CD8^+CD28^-CD27^-$ subsets are characterized by the most powerful effector activities. Their effectors and cytotoxity seem to be similar to the effector function (T_E, perforin and IFN-γ) of CTLs in this study. Consequently, it seems likely that CTL-mediated defence plays a crucial role against alloantigen-primed T cells, viral infection and microbial infection.

In addition, it was suggested that the administration of donor lymphocytes and recipient anti-donor lymphocyte antibodies prior to organ transplantation brings about continuous alloantigen elimination in rats [27]. We could not evaluate those events after LDLT.

Until now, beneficial effects of Tac/MMF have been reported on clinical outcomes and survival rates after liver transplantation [28–30]. However, those follow-up studies were performed for deceased donor liver transplantation, and have not been performed for selected HPT_E recipients after LDLT.

Finally, the addition of MMF to Tac-based immunosuppression (steroid-free) in HPT_E recipients enhanced the effector function of CTLs effectively, and improved clinical outcomes associated with the longest survival. The highest expression of IL-12Rβ1^+ T_{CM} ($+T_{DP+}$) linked closely to the steady-state expression of IL-12-Rβ1^+ T_N may play a crucial role in preventing the development of various post-transplant episodes.

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Disclosure

The authors declare that neither the submitted material nor portions thereof have been published previously or are under consideration for publication elsewhere. There are no financial and commercial conflicts of interest.

Author contributions

T. K., A. M., Y. E. and S. U. performed living donor liver transplantation for all recipients. S. U. and K. O. designed the research study. K. O. analysed all the data statistically. S. U. and K. O. wrote the paper.

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